the junction of primary and secondary cell walls, there is generally an abrupt change of direction.

The wax canals observed in the freeze-etch replicas consist mainly of transverse cross sections or short longitudinal fractures. Since they are distributed throughout the cell wall and are often observed entering from the cuticle and plasmalemma, there is little doubt that they traverse its full width.

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17 July 1967

Effect of Amantadine Hydrochloride on the Response of Human Lymphocytes to Phytohemagglutinin

Abstract. *Amantadine hydrochloride (Symmetrel), which is an antiviral agent marketed for the prevention of infection by influenza virus, inhibits the mitogenic response of human lymphocytes stimulated with phytohemagglutinin. The concentrations which effectively inhibited the response to phytohemagglutinin were similar to those which maximally inhibit virus replication. The drug inhibited the mitogenic response without affecting the ability of phytohemagglutinin to agglutinate leukocytes. The data suggest that phytohemagglutinin, amantadine, and certain lipid-containing RNA viruses take part in cell-membrane interactions of common nature.*

Phytohemagglutinin (PHA), a mucoprotein obtained from *Phaseolus vulgaris*, agglutinates erythrocytes and leukocytes and initiates a mitogenic response of lymphocytes. In the presence of PHA, 60 to 90 percent of the lymphocytes undergo blastic transformation, with the maximum response observed in 3 days. Exposure of lymphocytes to a specific antigen results in blastic transformation of up to 30 percent of the cells, and this response requires about 6 days. The response to PHA of lymphocytes from infants with congenital rubella was impaired during the early course of the disease (*1, 2*). In vitro, addition of rubella virus to lymphocytes from normal subjects similarly impairs the response of the cells to PHA (*2*) and specific antigens (*3*).

Amantadine (1-adamantanamine HCl) is one of the first antiviral drugs marketed for the prevention of viral diseases (Symmetrel). The antiviral activity of the drug is limited to certain lipid-containing viruses (*4–10*). Amantadine exerts an antiviral effect by preventing virus penetration into the cell and not by altering virus adsorption or other phases of virus replication (*7, 11*). During the attempt to determine whether penetration of rubella virus was necessary to inhibit the PHA response of lymphocytes, it was found that amantadine also inhibited the PHA response of lymphocytes. The inhibition of the mitogenic response of the lymphocytes was not associated with a loss of the leukoagglutinin effect caused by PHA.

Heparinized blood samples were obtained from normal adult donors; the erythrocytes were allowed to settle at 37°C, and the leukocyte-rich plasma was decanted. The leukocytes were sedimented by centrifugation, washed once in Hanks's balanced salt solution, and again suspended to a final concentration of 0.5 to 1×10^6 nucleated cells per milliliter in Eagle's medium containing 20 percent fetal bovine serum, antibiotics (100 units of penicillin and 100 μg of streptomycin per milliliter), and 1.5 g of bicarbonate per liter. Cultures containing 2 ml each were prepared in quadruplicate. Phytohemagglutinin-M (0.05 ml) (*12*) was added to appropriate cultures. Stock solutions of amantadine HCl (*12*) (1000 μg/ml), prepared in distilled water, were used fresh or were frozen at −20°C until used. Further necessary dilutions of the drug were made in the culture medium. All cultures were incubated for 3 or 5 days at 37°C before 0.06 or 0.2 μc of H^3-thymidine (specific activity, 2 c/mmole) was added to three culture tubes of each set. Five hours later, growth of the cultures was terminated by the addition of an excess of unlabeled thymidine, and the cells were separated by centrifugation. The cells were washed in cold Hanks's solution, cold 5 percent trichloroacetic acid, and cold methanol. The final precipitate was dissolved in 0.1 ml of Hyamine (*12*) mixed with scintillation fluid and was counted in a Beckman liquid-scintillation counter. Data represent the average number of counts per minute for three cultures of each set. The fourth culture was used to determine cell morphology and viability by the dye-exclusion method (*13*). The source, preparation, and assay of rubella virus have been described (*2*).

Amantadine effectively inhibits rubella virus replication (*5, 8, 9*); we performed experiments in which amantadine (final concentration, 50 μg/ml) was added to leukocyte cultures 30 minutes before the cultures received PHA with or without rubella virus (Table 1). There was a reduction of thymidine incorporation of 50 to 60 percent when rubella virus was added to the leukocyte cultures containing PHA. Amantadine alone, when added to the cultures, produced a 60- to 70-

Table 1. Effect of amantadine and rubella virus on the lymphocyte response to PHA (at concentration of 1:50). Leuko-agglutination was arbitrarily graded 0 to +2, with +2 being maximum agglutination.

PHA	Amantadine	Rubella virus	Count/min*	Transformation (%)	Leuko-agglutination	Viable (%)
			Trial 1			
			181	4	0	95
	+		371	5	0	97
		+	81	1	0	100
+			6,443	64	+2	91
+		+	3,375	40	+1	92
+	+		1,901	40	+2	93
+	+	+	2,116	30	+1	96
			Trial 2			
			121	5	0	93
	+		105	7	0	89
		+	151	3	0	90
+			14,736	86	+2	92
+		+	5,590	69	+2	90
+	+		5,606	51	+2	89
+	+	+	3,639	44	+2	94

*H^3-thymidine (0.06 μc) added 5 hours before termination of cultures.

Table 2. Effect of amantadine concentration on the inhibition of the lymphocyte response to PHA.

Amantadine concentration (μg/ml)	PHA	Count/min*	
		Trial 1	Trial 2
100	+	267	61
50	+	257	64
25	+	261	1,475
10	+	24,053	36,636
5	+	42,780	23,824
1	+		27,596
	+	51,805	13,542
100		163	56
		337	369

*H^3-thymidine (0.2 μc) added 5 hours before termination of cultures.

percent reduction in incorporation of thymidine. Addition of both rubella and amantadine did not result in more inhibition than that observed in cultures containing amantadine alone. Thus, both rubella virus and amantadine effectively inhibited the mitogenic response of lymphocytes to PHA, but the inhibition does not appear to be additive. It seems likely that amantadine and rubella virus inhibit the PHA response of lymphocytes at different cellular sites, and that the amantadine acting at the cell surface prevents rubella virus from reaching an essential site (*2*).

Agglutination of leukocytes in cell preparations was not inhibited by either amantadine or rubella virus (Table 1). When 100 μg of amantadine per milliliter was added to cultures containing five times as much PHA, again there was no loss of the leuko-agglutinating property of the PHA.

The optimum time for the addition of amantadine was studied by adding the drug to give a final concentration of 50 μg/ml at intervals after the initiation of the cultures. Inhibition of thymidine incorporation was greater than 90 percent at all times tested (0, ½, 1, 2, 3, and 24 hours).

The concentration of amantadine needed to inhibit the mitogenic response of leukocytes to PHA was determined by adding various concentrations of the drug to leukocyte cultures simultaneously with PHA (Table 2). A reduction of 90 percent or more in thymidine incorporation was observed at concentrations of 25 μg/ml or greater, but not of 10 μg/ml or less.

The inhibition of the mitogenic response of leukocytes to PHA by amantadine is apparently not a toxic effect. This is indicated by the normal viability of the lymphocytes cultured in the presence of the drug (Table 1). In tissue culture systems, retardation in cell-growth rates and vacuolization of cells when the drug was used at concentrations of 50 to 100 μg/ml have been reported (*4, 8, 9*). No discernible toxicity has been found at 25 μg/ml, a concentration of amantadine that inhibits the mitogenic response to PHA.

Studies in vitro indicate that amantadine's role is the prevention of virus penetration (*11*). Whether this occurs by preventing pinocytosis or by altering the cell-membrane function in some other way is unknown. The concentrations of amantadine which effectively inhibit the mitogenic effect of PHA are the same as those found by others to inhibit viruses effectively; this suggests that the drug may inhibit PHA and viruses by a similar mechanism. If this is true, our findings may be construed as further evidence that PHA acts primarily at the cell surface to stimulate lymphocyte mitogenesis (*14*).

The fact that amantadine at concentrations of 100 μg/ml did not inhibit the leuko-agglutinating activity of PHA may be considered analogous to the lack of effect on virus adsorption observed with the drug. The ability to dissociate the leuko-agglutinating and mitogenic properties of PHA with amantadine confirms the observations of Börjeson *et al.* (*15*) who reported that lymphocytes coated with the Vi polysaccharide failed to agglutinate but that they did undergo blastic transformation in the presence of PHA. This analogy suggests that PHA, a macromolecule, may share certain properties with amantadine-sensitive viruses. This property (or properties) allows adsorption of PHA to the cells and agglutination, but, in the presence of amantadine, the cellular effects of PHA are prevented. Further investigation of the interaction of PHA, amantadine, and lymphocytes is obviously warranted.

Taken orally, amantadine is well adsorbed and excreted primarily in the urine. Concentration of the drug in the blood after the recommended oral dosage is far below that which is necessary to inhibit the mitogenic effect of PHA on lymphocytes in vitro (*16*). However, amantadine in vivo may partially inhibit the lymphocytes undergoing mitosis after an antigenic stimulation. Under the influence of amantadine, the amounts of antibody produced in response to an antigen may be reduced. In a clinical trial during an influenza epidemic, the group of patients on amantadine had fewer individuals with a fourfold rise in antibody titers to influenza virus. Of the patients on amantadine who had a fourfold or greater rise in antibody titer, the mean titers were lower than in the control groups (*17*). The lower concentration of antibody is probably due to decreased amounts of influenza antigens produced in the presence of the inhibitor; however, the amantadine may have partially inhibited the mitogenic response of the immunoglobulin-synthesizing lymphocytes which resulted in decreased production of antibody. The specific antibody response to nonreplicating antigens, as well as the effect of amantadine on the mitogenic response in vitro to substances other than PHA, must be studied before this point can be resolved.

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18. Aided by PHS grants AI-05382, HE-05435, HE-02085, 1-K3-AI 25,943, and GRS-FR-0385, and by grants from the National Foundation, the Minnesota Heart Association, and the Minnesota Division of the American Cancer Society. P.B.D. is a Leukemia Society Scholar.

7 August 1967